SYMPOSIUM ON AUTOTROPHY[1]

II. The Comparative Physiology of Autotrophic Bacteria

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I am somewhat in the situation described by Leacock, who pointed out that, being interested in education, he made a special visit to Oxford in 1921. He arrived at 4 in the afternoon and left at 11 the next morning. Since he had already similarly visited Oxford in 1907—his views were based upon observations extending over 14 years. My colleagues, LePage, O'Kane, and Vogler, and I studied a particular autotrophic bacterium over 20 years ago. I have only recently returned to its study.

This return has emphasized to me at least three aspects of our science, which I would have possibly missed in a more gradual development. First, over this interval there has been the development of methods for the precise and convenient study of biological problems. It is difficult today for a young research worker to realize what study was like, say, 20 years ago. There were no Beckman spectrophotometers, no paper chromatography—one had to isolate and identify by derivatives—there were no isotopic techniques. When one returns today to the same problem left two decades ago, he is impressed by how precise and how rapid and convenient are the present laboratories. Today we can do in a day what used to take us a week. This is not to say that the methods are free from error, but only that we can reach our erroneous conclusions sooner.

Second, our metabolic concepts were only rudimentary. The Meyerhof-Embden system had just been proposed, but it was doubtful that it existed in bacteria. The monophosphate shunt was just that—a "shunt," a side issue—if indeed it existed at all. The fixation of carbon dioxide by heterotrophs was possible but controversial, and the concept of phosphate bond energy had just been proposed. With respect to autotrophs themselves, there were certain elements of confusion which will not pay us to examine.

Third, I have been impressed by how the generations differ from each other: how evidence acceptable as proof to one generation is regarded as not pertinent by another. Perhaps because of the situation in which I happen to have fallen, I might become a bridge between the generations. Comparative physiology of autotrophic bacteria means to me not only the comparison of autotroph to heterotroph, or autotroph to autotroph, but the concept of autotrophy as we knew it then, and as we know it now.

There is, I regret to say, one area in which, in my opinion, there is a loss of knowledge of autotrophy. This arises because definitions have become fuzzy. If then we first take up the question "What are autotrophic bacteria?" we find that we are less clear today. This is not purely a matter of semantics. Winogradsky in 1887 (39) defined autotrophs as organisms capable of the synthesis of all of the organic material of the living cell independent of the rays of the sun. This is a very clever definition. If you regard glucose as dependent upon the rays of the sun, it eliminates heterotrophs. It eliminates photosynthetic organisms. Workers for the next 50 years were able to sharpen the definition somewhat. In 1947, 60 years after Winogradsky, the definition had two parts (32) and we recognized "strictly" autotrophic bacteria and "facultative" autotrophic bacteria. For strictly autotrophic bacteria, the sole source of energy for growth was the oxidation of an inorganic material. The second part of the definition was that carbon dioxide was the sole and irreplaceable source of carbon for growth.

By this rather rigid two-part definition, one could delineate a group of organisms—unusual, distinct, and of considerable interest. Such organisms did indeed exist. But what has happened in the intervening years? Here is a quotation from an essay on autotrophy, in 1954 (9):

[1] This symposium was held at the Annual Meeting of the American Society for Microbiology, Chicago, Ill., 24 April 1961, under the sponsorship of the Division of Agricultural and Industrial Bacteriology, with Robert L. Starkey as convener.

"No generally accepted definition of an autotrophic bacterium has yet been propounded. According to Umbreit, a true autotrophic bacterium can obtain energy only by the oxidation of a specific inorganic substrate and relies exclusively on carbon dioxide as a source of carbon. Such a definition unquestionably excludes a considerable proportion of those bacteria commonly regarded as autotrophic including evidently the photosynthetic groups and indeed only thiobacillus, and the true nitrifying bacteria appear to qualify. In this survey, however, we have examined all those groups to which the term autotrophic is normally applied at the present time, including the photosynthetic bacteria and those credited with obtaining some part of their energy from the oxidation of iron, sulfur, hydrogen, nitrogen, and carbon monoxide. As at present accepted, the autotrophic bacteria also include a variety of other organisms, some of which almost certainly have no autotrophic facilities, whereas others are not bacteria."

I don't think that we have made any progress in defining the subject by the application of such diffuse concepts.

As you know, Lees (17) has written a nice book on autotrophic bacteria, and he starts with a good point, namely, that the word autotroph means self-sufficient. But self-sufficient, when examined, proves to have a very fuzzy meaning. Lees points out that perhaps the best definition of a self-sufficient organism is one that can live, grow, and reproduce in an environment free from living organisms or of compounds made by them. But the most common such creatures are green plants. He then defines the autotrophic bacteria not on this basis at all, but upon the basis that the sole source of carbon is CO_2, which also includes the photosynthetic organisms. Without laboring the point further, the definitions and connotations, that have grown up around and fastened themselves, barnacle-like, to the concept of autotrophy, have in the past decade tended to confuse the issue. Winogradsky knew precisely what he meant by an autotroph and I shall stick with him.

There exist bacteria, albeit only a few, that can oxidize an inorganic source of energy which is their only source of energy for growth. In addition, in these same bacteria only carbon dioxide will serve as a carbon source for growth. These are "strictly autotrophic" bacteria; they exist, they are clearly distinguishable.

To simplify still further, we can specify that there are four clearly established groups of strict autotrophs as follows: the genus *Nitrosomonas*, oxidizing ammonium ion; the genus *Nitrobacter*, oxidizing nitrite ion; *Thiobacillus thiooxidans*, the acid sulfur oxidizers; and *Thiobacillus thioparus*, the alkaline thiosulfate oxidizers.

I think that there are justifiable reasons for separating these two latter species of organisms. It is my distinct impression that their basic physiology is different. It is only in the alkaline thiosulfite oxidizer, *T. thioparus*, that one begins to find facultative autotrophs. In all other groups there are only strict autotrophs. As such, I definitely exclude the photosynthetic bacteria from the autotrophs. I do not say that they are not related, or even that they are uninteresting, but I say only that they are not strict autotrophs. If we look at this small core of organisms and clearly understand them, we may discern relationships to others, but any relationships should be established experimentally and not gratuitously assumed. Further, this core of strict autotrophs has some very exciting creatures in it and they are quite worthy of study in their own right.

To begin, the strictly autotrophic bacteria attack an inorganic substance and oxidize it to obtain energy. Our first question is, how is it possible for them to derive energy from an inorganic substrate? There are really two problems here. First, one of the substrates, sulfur, is so insoluble that, before isotopes, no one had been able to measure its solubility. How does it get into the cell? Second, once in, how are such inorganic materials oxidized?

With respect to sulfur and its oxidation by *T. thiooxidans*, we had thought that this problem was solved by the demonstration that a direct contact between the sulfur and organism was necessary (36). We further supposed that the bacterium contained a solvent for the sulfur, in the form of a fat globule capable of dissolving sulfur (34). In its day this was evidence enough; but no longer. What has happened is that Starkey, Jones, and Frederick (26), Newburg (19), and others have demonstrated that one can obtain good growth on sulfur on a shaking machine, under which conditions one might suppose that contact between the bacteria and the sulfur would be easily broken.

We have recently studied growth on shaking

machines (incidentally, I might point out that 20 years ago, we never thought of using a shaking machine for aeration) and find that shaking does indeed inhibit the sulfur bacterial contact, but once this contact is established, it is surprisingly firm. As a working hypothesis we have supposed that the bacteria bore themselves a little hole in the sulfur crystal and when sitting in this are dislodged only with difficulty.

But once in, how are inorganic materials oxidized? For sulfur we know only that the respiratory system is a metal porphyrin and that sulfur is somehow converted to electrons passed along it (38). But how this is done is not clear. The cytochromes of the alkaline thiosulfate oxidizers have been isolated but seem to differ slightly from animal cytochromes (6, 15, 18, 29), and the cytochromes from *T. thiooxidans* have proved to be difficult to obtain (29). For ammonia, there are now available enzymatic systems with electron transporting properties (11, 13); for nitrite, there is available a cell-free system, capable of nitrification (1, 2, 14). For thiosulfate there are similar preparations. Since Lees will discuss subsequently in this symposium the biochemical paths of such primary reactions, I shall not mention them further except to point out that they are all devices for abstracting electrons from the inorganic material and that the energy is generated by the passage of these electrons through systems at least comparable to, if not identical with, systems found in heterotrophic creatures.

Perhaps the second main question one might ask about autotrophs is "what kinds of cells are these?" I shall dispense with any debate on their alleged primitive nature; today it is unimportant. But one may say that the cells themselves are much like those of heterotrophs, even though they have all been made from CO_2. They contain similar enzymes and vitamins. As a matter of fact, *T. thioparus* has been used to prepare C^{14}- and CO^{60}-labeled vitamin B_{12} (20); *Thiobacillus denitrificans*, to prepare C^{14}-labeled ribonucleic and deoxyribonucleic acids, 2-deoxyribose, and thymidine (8); and *T. thiooxidans*, for the preparation of amino acids (12). They contain an array of phosphorylated intermediates. In short, today's evidence is clear that strictly autotrophic bacteria possess, within the cells themselves, a heterotrophic metabolism.

But, if so, why do they not use organic materials? The answer is that, in part, they do use organic materials. Starkey, as early as 1925, (25) showed quite clearly that *T. thiooxidans* could slowly use glucose but it could not *grow* on this energy source. There are some more data of this sort, but many of them are unusable because of the unrecognized easy contamination of autotrophic cultures. However, admittedly, the use of organic materials is marginal at best, and we may rephrase our question to be: why do they not use organic materials at all readily? In this connection, it is my supposition, supported mostly by inference, that the strict autotroph is adapted to life in what is essentially a toxic environment and that it has therefore so changed its permeability properties that all but a very few materials are excluded. In short, it is the biological submarine living in a hostile world by excluding it. I am not quite sure what would constitute proof of this assertion, but in the meantime I hold firmly to this delusion.

When we first uncovered the relationship between sulfur oxidation, phosphate, and carbon dioxide fixation (37), it was in the very early years of the energy-rich phosphate bond concept. We may be pardoned an enthusiasm for it. The demonstration of "energy storage" in *T. thiooxidans* by Vogler and myself (37) has been, so far as I am aware, unique, and does not seem to be found in other systems. The data have been criticized and I think that there is indeed an error in this work, in that, because of an odd relation between cell nitrogen and turbidity about which we did not know at the time, we had probably as much as five times as many cells in our respirometer as we supposed. It happens that it takes many more young cells to reach the same turbidity achieved by fewer old cells. On this basis, the amount of carbon dioxide fixed and organic phosphate formed and released is just about what one can find today (33) with the more comfortable and accurate isotopic methods, and is not impossible as calculated by the Baalsruds (7) and by Vishniac and Santer (35). But aside from certain controversies, what has been clearly established in the intervening years is that the result of the oxidation of the inorganic energy source is a source of energy-rich phosphate to the cell. It is not yet clear, nor indeed was it ever clear, that the energy-rich phosphate was the only thing

that the cell obtained from its oxidation, but this was the simplest assumption and it has remained our working hypothesis.

If we conceive of such cells as walling away the world, and allowing only a few choice substances to penetrate, do they have a different or a similar way of fixing and reducing carbon dioxide when compared to photosynthesis? There had been much speculation on this point, and it seemed so obvious to many that they felt that experimental demonstration was superfluous. But what is obvious to one person is frequently not obvious to another. Twenty years ago, believe it or not, formaldehyde was still a serious contender as an intermediate in photosynthesis. It always seemed to us that if there were a relation between chemosynthesis and photosynthesis, this relation would need to be established experimentally. As known to us in *T. thiooxidans*, there was a phosphate bond generated and this was involved in the fixation of carbon dioxide. We thought that a similar relation might exist in photosynthesis. We could not find any evidence of energy storage, but we could demonstrate plenty of evidence for phosphate changes (10). There is a curious irony here. We were, of course, looking for some energy-rich phosphate bond, but all that we could find was phosphoglyceric acid. Without isotopes, neither we, nor anyone else, had the least suspicion that this substance had anything to do with photosynthesis.

Insofar as it has been studied, the pathways of CO_2 fixation in autotrophs seem to be similar to those of photosynthesis. Santor and Vishniac (23) obtained extracts from *T. thioparus* in which ribulose diphosphate fixed CO_2 to form phosphoglycerate, and similar results were obtained by Trudinger (30, 31) and by Aubert, Milhaud, and Millet (4, 5) in *T. denitrificans*. Kornberg, Collins, and Bigley (16) have even demonstrated that in *T. denitrificans* the CO_2-fixation system via ribulose diphosphate is adaptive and organisms grown on various carbon sources, rather than on thiosulfate, differ in the pathway used to fix CO_2. Suzuki and Werkman (27, 28) in cell-free extracts of *T. thiooxidans* demonstrated that there is a fixation of carbon dioxide into the dicarboxylic acids by way of the Wood-Werkman system.

However, at this state of our information, the data available to me support a curious conclusion, one which, indeed, I do not suppose in the long run will prove to be correct. This conclusion is that the thiosulfate oxidizers and more particularly the facultative types fix a large part of their carbon dioxide via the addition of carbon dioxide to ribulose diphosphate forming phosphoglyceric acids (4, 5, 16, 23, 30, 31), whereas the acid sulfur oxidizer, *T. thiooxidans*, fixes carbon dioxide via CO_2 addition to phosphopyruvate (27, 28).

There is, then, a relation between chemosynthesis and photosynthesis and this relation consists, so far as we can now discern, in a striking similarity in the pathway of carbon dioxide fixation and in the involvement of phosphate in the primary energy-yielding systems. It is the latter that has had a long and rocky road. Indeed, when we proposed that in photosynthesis the first step was the formation of energy-rich phosphate bonds (10), we were severely criticized and Rabinowitch (22) devoted a section in his book on photosynthesis to pointing out how wrong this kind of concept was and how it was a step in the wrong direction. Today there is clear evidence for the participation of energy-rich phosphate in the light-activated steps of photosynthesis (*see* Arnon, (3)), but naturally there is still much to be learned about the details of the process in both the autotrophic bacteria and in the photosynthetic organisms.

We have so far compared the physiology of the strictly autotrophic bacteria to the heterotrophs, but it is of some interest to make some comparisons among them. A cell-free system from *Nitrobacter agilis* (2) demonstrates phosphorylation coupled to the specific oxidation of nitrite. In *T. thioparus* the oxidation of thiosulfate, especially to tetrathionate, proceeds without the involvement of phosphate, but from there on, phosphate is required (24) and cell-free extracts from this organism form adenosine triphosphate and adenosine 5-phosphosulfate (21).

What I am trying to point out is that although the preliminary maneuvers are different with various autotrophs, there seems to be a degree of unity among them and it seems apparent that they employ the peculiar character of the energy-rich phosphate bond as an energy carrier.

It seems to me increasingly clear that even the strict autotrophs do not differ in basic mechanisms either from the heterotrophs or from each other, but it is in the ability to attack their original and peculiar energy substrate that they

are unique. These two factors, the ability to attack and to derive energy from an inorganic substance and to wall off virtually all the rest of their environment, seem to me to be the unique characteristic of autotrophic bacteria. The study of their comparative physiology has served over the past two decades not only to indicate their similarity to each other and their relation to heterotrophic life, but also to delineate those features which make them distinct and different—which is, indeed, the basis of autotrophy.

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